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Expression of maize *KNOTTED1* related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot

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SUMMARY

In this paper we describe the expression patterns of a family of homeobox genes in maize and their relationship to organogenic domains in the vegetative shoot apical meristem. These genes are related by sequence to *KNOTTED1*, a gene characterized by dominant neomorphic mutations which perturb specific aspects of maize leaf development. Four members of this gene family are expressed in shoot meristems and the developing stem, but not in determinate lateral organs such as leaves or floral organs. The genes show distinct expression patterns in the vegetative shoot apical meristem that together predict the site of leaf initiation and the basal limit of the vegetative 'phytomer' or segmentation unit of the shoot. These genes are also expressed in the inflorescence and floral meristems, where their patterns of expression are more similar, and they are not expressed in root apical meristems. These findings are discussed in relation to other studies of shoot apical meristem organization as well as possible commonality of homeobox gene function in the animal and plant kingdoms.

Key words: *KNOTTED1*, meristem, homeobox, *Zea mays*

INTRODUCTION

Development of the shoot system in plants proceeds through the elaboration of organs initiated on the flanks of the shoot meristem(s), which are situated at the apex of the shoot above the most recently formed leaf. How the components of the shoot, such as leaves, stem internode and axillary buds become organized from initially indeterminate cells in the meristem is unclear. This process is, however, a major determinant of plant form, since, for example, it establishes the phyllotaxy, or spatial arrangement, of lateral organs around the apex (Richards, 1951).

The vegetative shoot meristem has been extensively described histologically (reviewed by Steeves and Sussex, 1989; Medford, 1992). A zonation model describes the meristem by cytological characteristics (Ledin, 1954; Wardlaw, 1957), although the presence and extent of different cytological zones differs between species. In general, cells at the apex ('central zone') of the meristem are larger and thought of as initials, having some properties in common with animal stem cells (Sussex, 1989). Daughter cells of the initials replenish the subjacent organogenic region ('peripheral zone') which is characterized by cytoplasmically dense cells that are metabolically more active. Below this is the rib zone, composed of regular files of cells, which give rise to the stem. Meristems have also been described in terms of layering, since the periphery of the meristem consists of regular layer(s) of cells (Hanstein, 1868), called tunica layers, which surround the central corpus (Schmidt, 1924). Each tunica layer is clonally derived since it is replenished predominantly by anticlinal cell divisions (Satina et al., 1940). Though the number of tunica layers differs widely between plant species (e.g. Kaplan, 1970), in maize and other grasses there is generally only one, the protoderm (Esau, 1965).

Vegetative shoot development is a repetitive process whereby leaf primordia are successively initiated at the meristem. In maize, leaves are initiated singly on the flank of the meristem opposite to the previous leaf, resulting in a distichous phyllotaxy. A periclinal cell division in the tunica on the flank of the meristem precedes the initiation of a leaf primordium, and is closely followed by a reorientation of divisions in the corpus (Ledin, 1954; Esau, 1965). Clonal analysis has indicated that approximately 250 leaf founder cells are present at the time of leaf initiation (Poethig, 1984). As the new primordium enlarges, a wave of cell division spreads around the meristem circumference so that the base of the leaf encircles the meristem, and the disc of leaf insertion is formed (Sharman, 1942; see Fig. 1). Cells at the base of the disc of leaf insertion give rise to the internode (stem) and axillary bud (Sharman, 1942). Thus the vegetative shoot can be viewed as consisting of segments, each comprising of a leaf, leaf node, internode and axillary bud. These segments have also been termed phytomers (Galinat, 1959). Consistent with the histological observations, clonal analysis has shown that in maize the leaf is clonally related to the internode and axillary bud below it (Johri and Coe, 1982; McDaniel and Poethig, 1988), though this is not the case in all plants (e.g. Furner and Pumfrey, 1992; Irish and Sussex, 1992). During shoot ontogeny, the time interval between the initiation of leaf primordia is termed a plastochron (Askenasy, 1880). In addition, the plastochron index describes the position of leaf primordia in relation to the meristem (Lamoreaux et al., 1978).

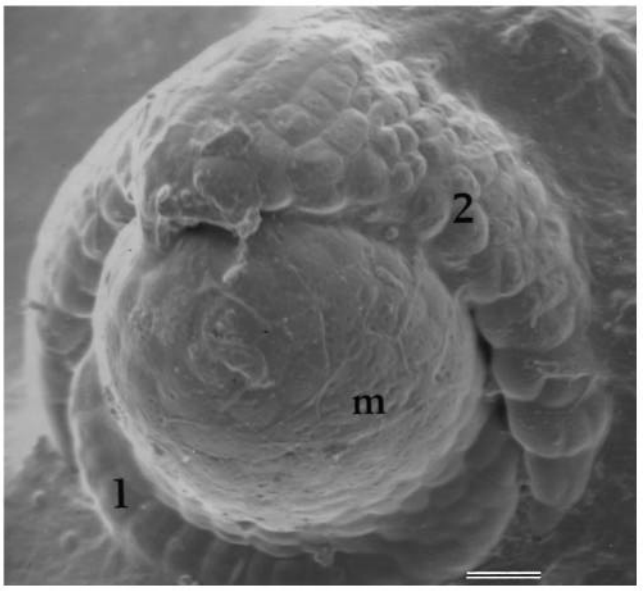

Fig. 1. The maize vegetative shoot apical meristem. Scanning electron micrograph of the shoot apex from which most of the leaves have been removed, viewed from above. The youngest, P_1 leaf and the older P_2 leaf are visible. Note that the base of each leaf ('the disc of leaf insertion') encircles the shoot apex. The predicted position of the P_0 or incipient leaf is on the flank of the meristem under the P_2 leaf, i.e. opposite the P_1 leaf. Photograph provided by T. Foster. m, shoot apical meristem; 1, 2, plastochron 1, 2 leaf. Bar, 20 μm.

The youngest primordium is thus referred to as the plastochron 1 or P_1 leaf, with successive older leaves being P_2, P_3 etc. (Fig. 1). The position of the incipient leaf is referred to as plastochron zero or P_0.

Despite the many studies of vegetative meristems, there is a paucity of molecular markers with which one could probe their structure (e.g. Fernandez et al., 1991; Medford et al., 1991; Pri Hadash et al., 1992; Fleming et al., 1993). An insight into the organization of meristem domains in maize has come from studies on the *KNOTTED1* gene. *KN1* is characterized by a series of dominant mutations which affect the development of the leaf blade; mutant leaves show localized zones of extra cell division in all cell layers, resulting in outgrowths or knots (Bryan and Sass, 1941). *KN1* encodes a homeodomain protein that is, however, expressed at only very low levels in leaves of wild-type plants (Vollbrecht et al., 1991; Smith et al., 1992). It is expressed strongly in the meristem and in ground tissue of the unexpanded stem, but is absent from the predicted position of the incipient or P_0 leaf in the meristem. Ectopic expression of KN1 in leaf veins of mutant plants correlates with the presence of the knotted phenotype. Based on these observations it was proposed that the *KN1* gene product is involved in the maintenance of the indeterminate state of plant meristems, and that its down regulation leads to the initiation of determinate lateral organs such as leaves or floral organs (Smith et al., 1992).

We were interested in the possibility that other maize homeobox genes in addition to *KN1* might be useful markers for meristem organization. The homeobox encodes the homeodomain, which is a DNA binding domain (Pabo and Sauer, 1984; Desplan et al., 1988), and it has been shown in many organisms that related homeobox genes have similar functions and specify important determination events in development (Gehring, 1987; Ingham, 1988; McGinnis and Krumlauf, 1992). A family of *KN1* related genes was therefore isolated from maize (Vollbrecht et al., 1991, 1993), and has been named KNOX, for *KNOTTED* related homeobox (Kerstetter et al., unpublished data). The expression patterns of *KN1* and three related homeobox genes are described here. One of these genes has been shown to correspond to the dominant leaf mutation *ROUGH SHEATH1* (Freeling, 1992; Becraft, Schneeberger, Hake and Freeling, unpublished observation). The results presented here for *RS1* expression have also been confirmed elsewhere (R. Schneeberger, personal communication). We show that, like *KN1*, the three other maize homeobox genes are expressed in shoot meristems and in the developing stem, but not in determinate lateral organs. This collection of probes defines regions in the vegetative shoot apical meristem whose positions predict the site of leaf initiation and the basal limit of the vegetative segmentation unit, and should be useful tools to explore morphogenetic events in the meristem.

MATERIALS AND METHODS

Probes

The probes were prepared by in vitro transcription with digoxigenin-labeled UTP (Boehringer Mannheim) according to manufacturers instructions, except the ratio of labeled to unlabeled UTP was 1:1. Probes were purified and hydrolyzed as previously described (Jackson, 1991). The clones used were complete or partial maize cDNAs, in some cases subcloned as small fragments to verify that the same expression pattern was obtained when using conserved and non-conserved parts of the cDNA as a probe.

(1) *KN1*, 3 subclones (0.4-0.65 kb, the conserved homeobox region was contained within one of the probes) of the 1.6 kb cDNA clone were transcribed separately, and used alone or as a mixture.

(2) KNOX8 (previously called cR18), a 0.6 kb cDNA clone.

(3) *RS1* (previously called ECB), a 0.8 kb cDNA clone.

(4) KNOX3 (previously called ECA), two cDNA subclones of the cDNA (0.6 kb *Aat*II-*Nae*I fragment and a 0.3 kb *Nae*I-*Xho*I fragment, from the middle (including the homeobox) and 3′ part of the cDNA, respectively).

(5) ubiquitin, a 0.65 kb *Pst*I-*Sac*I subclone of a maize cDNA clone (Christensen and Quail, 1989).

Other aspects of the molecular characterization of the homeobox clones will be published elsewhere (Kerstetter, unpublished data).

Tissue preparation and in situ hybridization

All plants used were from the inbred line B73 (Pioneer Hi-bred International), except for those carrying the *Kn1* mutations, as described in the text; wild-type siblings from these lines were used as controls. Seedlings were grown for two to three weeks in a mist room then transferred to larger pots and grown in the greenhouse. Roots were taken from seeds germinated on damp tissues as well as soil grown seedlings. Tissue was fixed and embedded and used as described (Jackson, 1991), with the following modifications: (1) vegetative shoot apices were fixed in FAA (5% formalin, 5% glacial acetic acid, 45% ethanol) overnight at 4°C instead of in formaldehyde, (2) The high stringency post-hybridization washes were in 0.2 × standard saline-citrate buffer (1 × is 150 mM sodium chloride, 15 mM sodium citrate) at 55°C, (3) digoxygenin-labeled probes were used at a concentration of 0.5 ng/μl/kb probe complexity, and detected as described by Coen et al. (1990). Some sections were stained in basic fuchsin,

0.05% in water, and slides were dehydrated through an ethanol series, rinsed twice in histoclear (National Diagnostics), and mounted in Merckoglas (EM Science).

RESULTS

Expression of homeobox mRNAs in vegetative tissues

A large number of cDNA clones was isolated from seedling and immature ear cDNA libraries using the *KN1* homeobox as a hybridization probe (Vollbrecht et al., 1991, 1993; Kerstetter et al., unpublished data). The expression of four homeobox genes, *KN1*, KNOX8, *RS1* and KNOX3 is shown in median longitudinal sections through the shoot apex of 2-3 week old seedlings in Fig. 2. At this stage the meristem has initiated approximately ten to twelve leaves. The expression of all four genes is limited to the meristem and developing tissues in the stem; there is no detectable message in young leaf primordia or in older leaves. Control sense strand probes showed no hybridization signal (not shown) except for some faint staining over the oldest leaves, which was also seen with antisense probes and was interpreted as being non-specific. The localization of *KN1* mRNA (Fig. 2A) is similar to that described for the KN1 protein (Smith et al., 1992); specifically the meristem and developing stem are labeled with the exception of certain provascular cells associated with developing lateral vein traces in the stem (arrowed). KNOX8 is expressed in a similar pattern to *KN1*, throughout the meristem and developing stem, though the expression is weaker than *KN1* and there is no differential labeling of cells associated with provascular traces (Fig. 2B).

The genes *RS1* and KNOX3 show a more localized expression pattern in the vegetative shoot apex. Small groups of cells expressing these genes are observed near the base of each leaf (Fig. 2C,D). In the case of *RS1*, discrete spots of expression are also seen in the meristem, whereas KNOX3 usually shows diffuse staining throughout the meristem (see later). In transverse sections we found that the 'spots' are actually rings of expression which encircle the stem at each leaf insertion point (Fig. 2F,G). Since the maize leaf is attached all around the stem, these rings of expression correspond to the base of the disc of insertion of each leaf (Sharman, 1942). This is the region from which the clonally related internode and axillary bud develop (Sharman, 1942; Johri and Coe, 1982; McDaniel and Poethig, 1988). Indeed, near the base of older leaves we observed expression of *RS1* and KNOX3 in both the developing internode and axillary bud (Fig. 2H). *RS1* also labels provascular cells in the stem (arrowed, Fig. 2C); in adjacent sections we observed that these include the cells of the provascular lateral traces which are not labeled by *KN1* (not shown).

Detailed localization of *KN1*, *RS1* and KNOX3 expression in the shoot apical meristem is shown in Fig. 3. *KN1* mRNA is expressed throughout the corpus of the meristem with the exception of two groups of cells, one on either side of the meristem, above the P_1 leaf. We interpret these two groups of cells as belonging to a doughnut shaped ring of cells corresponding to the disc of insertion of the P_0 preprimordium. In support of this interpretation, periclinal cell divisions, the first indication of the new primordium, are visible in the tunica opposite and above the P_1 leaf. (arrowed). *KN1* mRNA is not detectable in the tunica layer of the meristem.

RS1 and KNOX3 expression was localized as either two spots or a stripe of expression just above the site of insertion of the P_1 leaf (Fig. 3B,C). As in the stem, we conclude that the cells expressing these genes are arranged in a ring which encircles the meristem; depending on the plane of the section this is visualized either as two spots, i.e. if the section is median, or as a stripe if the section is slightly off median and cuts through the edge of the ring of expression, as is the case in the example shown. Whereas all meristems probed with the *RS1* probe showed this pattern of spots or a stripe, the majority of meristems probed with KNOX3 showed lower, diffuse staining (e.g. see Fig. 2D).

By probing adjacent sections with *KN1*, *RS1* and KNOX3 we were able to align more precisely their regions of expression. We found that *RS1* and KNOX3 are expressed in the same cells at the base of the disc of leaf insertion and that these are within the domain of *KN1* expression (Fig. 2E-G, also seen in longitudinal sections (not shown)). This is also the case for the rings of *RS1*- and KNOX3-expressing cells in the meristem (not shown). Thus in the meristem, the ring of cells expressing *RS1* and KNOX3 is just below the ring of cells which do not express *KN1*.

Although there are characteristic differences between the expression patterns of *RS1* and KNOX3, we sought to rule out the possibility of probe cross hybridization. This was accomplished by competition experiments in which a ten-fold excess of unlabeled antisense RNA was included in the hybridization along with the labeled antisense probe. In each case the unlabeled RNA competed out only the signal from the same gene probe (not shown). In addition, for *KN1* and KNOX3 we observed the same patterns of expression using non conserved parts of the cDNA as with full length cDNA probes.

We found an intriguing difference between the expression of *KN1* protein and mRNA. Whereas the protein is detected in all cell layers of the meristem (Fig. 4B), we have never detected *KN1* mRNA in the tunica layer of vegetative, inflorescence or floral meristems (Figs 3A, 4A). Control sections probed for ubiquitin mRNA show staining in all cell layers (Fig. 4C), indicating retention and accessibility of mRNA is not impaired in the tunica.

Expression of homeobox mRNAs during inflorescence and floral development

After the vegetative apical meristem has initiated around 16-22 leaves (depending on the cultivar) it elongates and forms the terminal inflorescence (Russell and Stuber, 1983; Irish and Nelson, 1991). This will later develop into the tassel, bearing male florets, whereas axillary inflorescences will develop into ears which bear female florets. Inflorescence meristems initiate branch primordia; these bifurcate to form spikelet meristems which later produce floral meristems (Postlethwaite and Nelson, 1964). Initial development of tassel and ear inflorescences is similar, but at relatively late stages they can be easily differentiated by differences in branching as well as by selective abortion of floral organs (Cheng et al., 1983).

In serial sections through tassel (Fig. 5A-D) and ear (not shown) inflorescences we observed that all four homeobox genes are expressed evenly throughout the corpus of the inflorescence meristem. In addition, the four genes are expressed

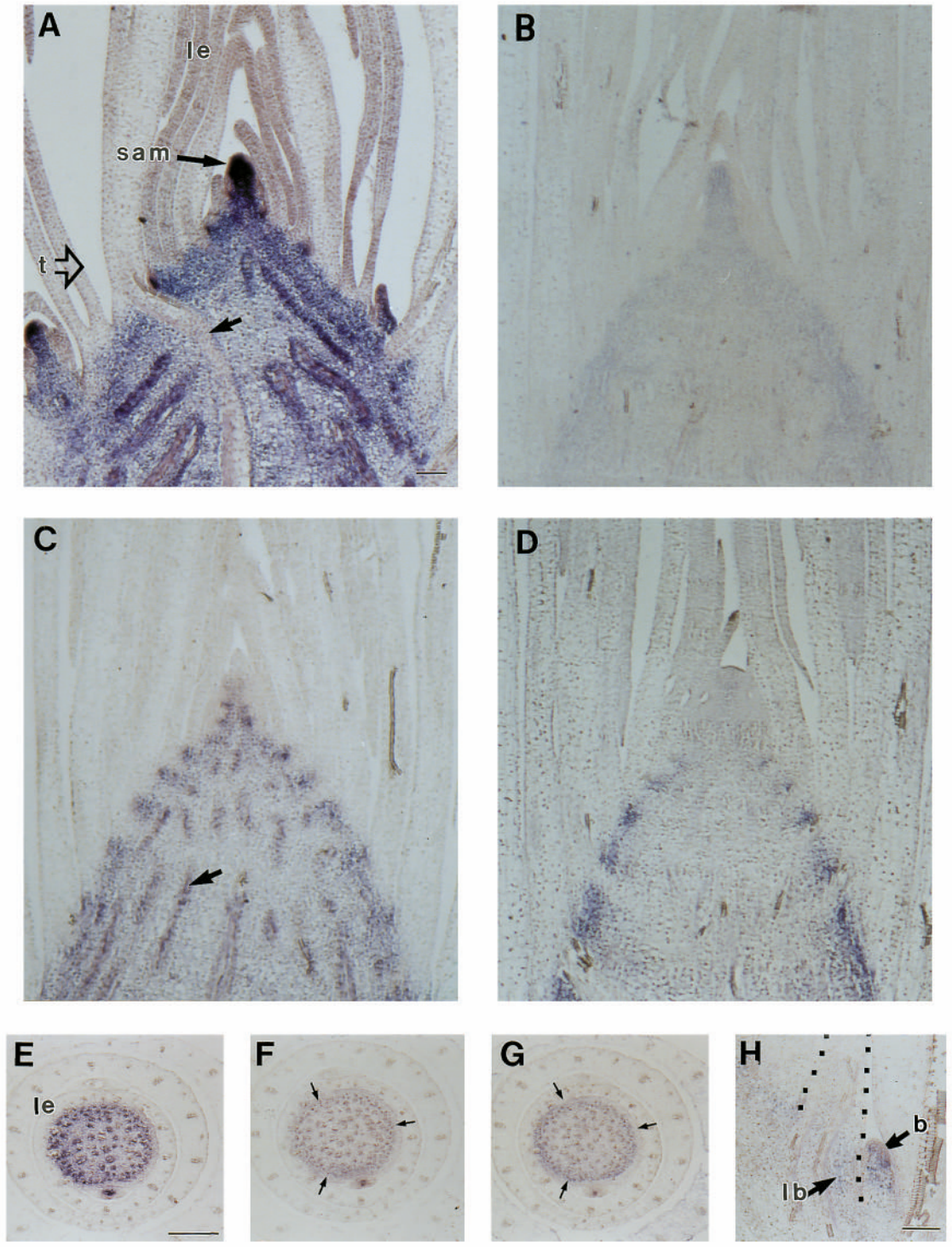

Fig. 2. Expression of homeobox genes in vegetative shoot apices. (A-D) Median sections through 2-3 week old seedling shoot apices, probed with (A) *KN1*, (B) KNOX8, (C) *RS1* and (D) KNOX3 antisense RNA probes. Hybridization is visualized as the blue coloured product of the alkaline phosphatase reaction. Bar, 100 μm. Note that *KN1* (A) labels the meristem and the ground tissue of the stem with the exception of some of the provascular strands (arrowed); *RS1* (C) labels the provascular strands in the developing stem, which are not labeled by *KN1* (arrowed). *RS1* (C) and KNOX3 (D) label groups of cells near the base of each leaf where it adjoins the stem. sam, shoot apical meristem; le, leaves. (E-G) Transverse sections through the stem, at the level of the P_5 leaf (arrowed (t) in A), probed with (E) *KN1*, (F) *RS1*, (G) KNOX3. The expression of *KN1* in E marks the stem, which is surrounded by leaves that are not labeled. Note that both *RS1* and KNOX3 are expressed in a ring around the outer part of the stem (arrowed) Bar, 500 μm. (H) Longitudinal section through the base of the seventh leaf from the meristem, showing the leaf (outlined by dots) and the developing axillary bud (b), probed with *RS1*. Note that expression is evident both in the axillary bud (b) and in cells at the leaf base (lb) which is beginning to elongate to form the internode. This same expression pattern was seen in sections probed with KNOX3 (not shown). Bar, 100 μm.

in spikelet and floral meristems, but are not expressed in lateral organs such as glume primordia. The discrete rings of expression of *RS1* and KNOX3 observed in the vegetative shoot apical meristem are not observed in inflorescence or floral meristems. In addition, *KN1* is highly expressed in certain developing vascular strands, and more weakly in the ground tissue of the inflorescence shoot. *RS1* and KNOX3 are expressed in the ground tissue of the inflorescence stem, though not expressed in those provascular cells labeled by *KN1* (Fig. 5A,C,D, arrowhead). Since these genes are expressed in all apical meristems of the shoot system, we were interested to see if they might also be expressed in meristems

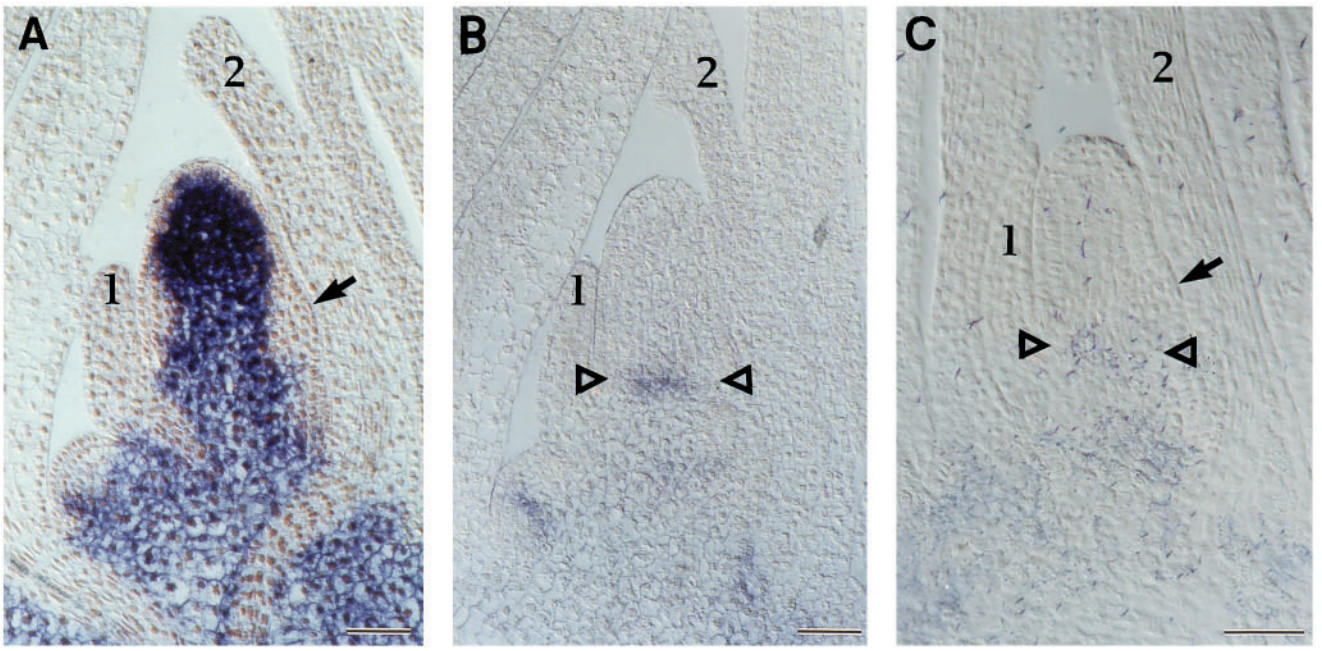

Fig. 3. Detail of homeobox mRNA expression in the shoot apical meristem. Sections through the shoot apex probed with (A) *KN1*, (B) *RS1* and (C) KNOX3. The central dome is the meristem, which is flanked by the P_1 and P_2 leaves (1 and 2). In A, the patch of cells not expressing *KN1* on either side of the meristem corresponds to the predicted position of the disc of insertion of the P_0 leaf. *RS1* (B) and KNOX3 (C) are expressed in a stripe (marked by arrowheads) just above the P_1 leaf. In sections A and C periclinal divisions in the epidermis, thought to precede primordium formation (Ledin, 1954; Esau, 1965), are visible (arrowed). Note that C shows a section through the shoot apex of an embryo, 22 days after pollination; the embryonic shoot meristem is a little smaller than the seedling meristems shown in A and B. Bar, 50 μm. Photographed using Nomarski optics.

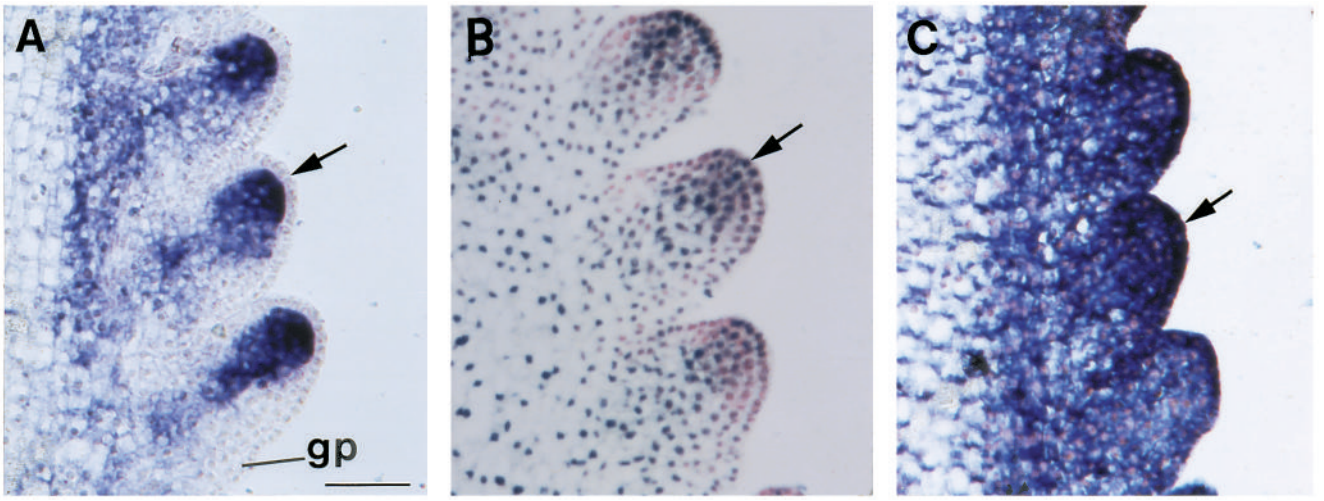

Fig. 4. KN1 protein and mRNA localization differs in meristems. (A) Section through three spikelet meristems on the flank of the ear inflorescence, showing in situ localization of *KN1* mRNA; note strong staining in the corpus but absence of staining from the tunica (arrowed), and from the glume primordia (gp) initiating on the flanks of the meristems. (B) Localization of KN1 protein, as described in Smith et al. (1992); using an affinity purified polyclonal antibody, visualized with a secondary gold-conjugated antibody and silver enhancement and seen as black staining over the nuclei (figure reproduced from Smith et al., 1992). Note glume primordia are not labeled, however nuclei in the tunica layer of the meristem are labeled (arrowed). (C) In situ localization of ubiquitin mRNA shows equal expression in all cells, including those of the tunica (arrowed) and glume primordia. Bar, 50 μm.

of the root system. However, none of the homeobox genes is expressed in primary (Fig. 5G-J) or secondary (not shown) root apices.

Expression of homeobox genes in knotted leaves

The mutant phenotype of the knotted mutation *Kn1*-N2 is caused by ectopic expression of KN1 in developing lateral

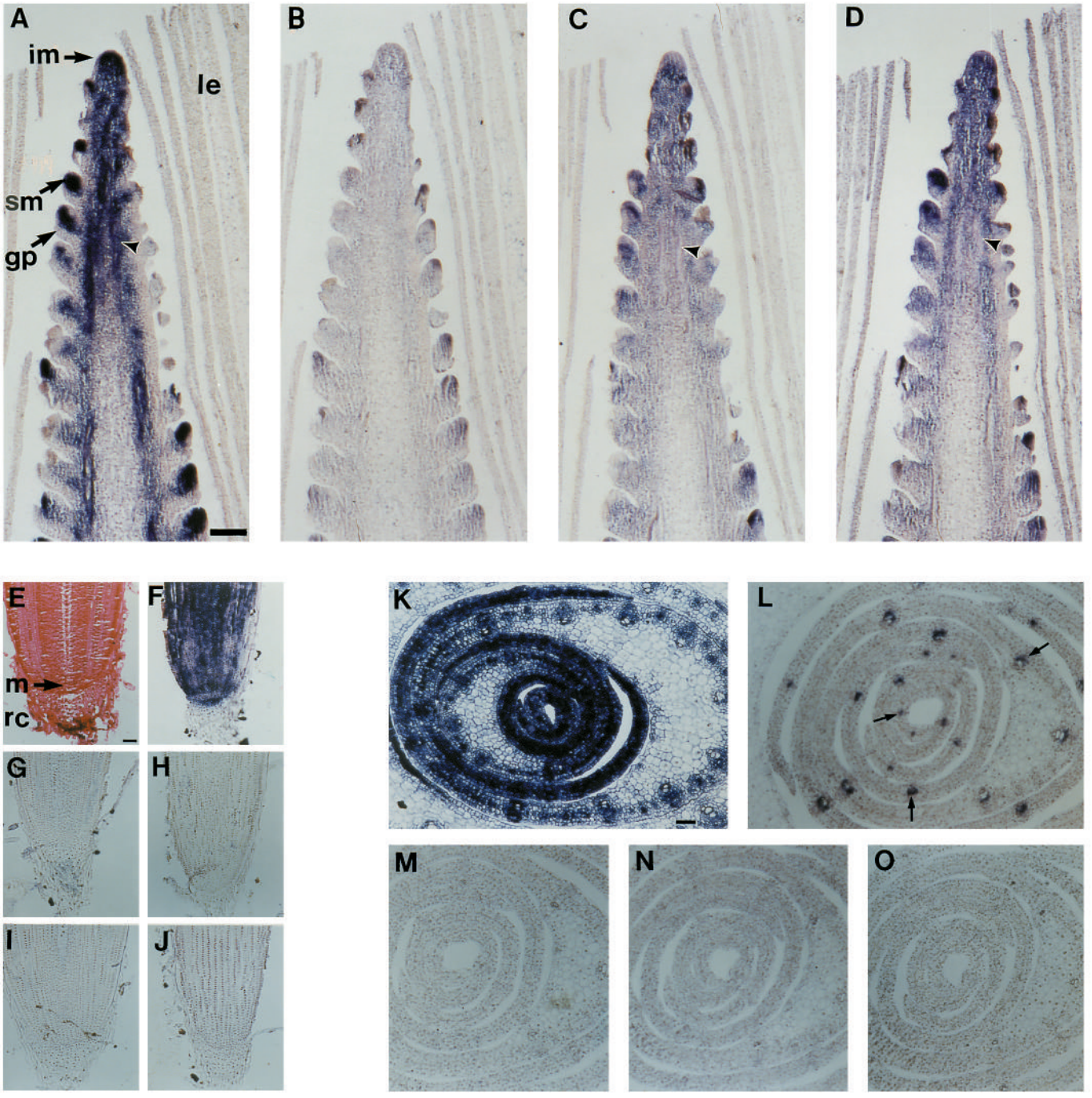

Fig. 5. Expression of the homeobox genes in the tassel inflorescence. (A-D) Serial longitudinal sections through a developing tassel inflorescence. Sections probed with *KN1*, KNOX8, *RS1* and KNOX3 respectively. Note all are expressed uniformly in the inflorescence and spikelet meristems. In the inflorescence stem, *KN1* (A) is expressed most strongly in developing vascular strands, whereas *RS1* (C) and KNOX3 (D) are expressed in the ground tissue of the stem and not in the vascular strands labeled by *KN1* (arrowhead). im, inflorescence meristem; sm, spikelet meristem; gp, glume primordium; le, leaves. Bar, 200 μm. (E-J) None of these homeobox genes is expressed in the root apical meristem. Longitudinal sections through primary seedling roots. (E) Roots stained with safranin, m, root apical meristem; rc, root cap. (F) In situ localization of ubiquitin mRNA, which is expressed throughout the root apex, with the exception of the root cap. (G-J) Sections probed with *KN1*, KNOX8, *RS1* and KNOX3 respectively; no hybridization signal is seen for any of these. Bar, 50 μm. (K-O) Expression of homeobox genes in knotted leaves. Transverse sections through seedling leaves, approximately 2 mm above the shoot apical meristem. (K) In situ localization of ubiquitin mRNA shows expression in all cell types. (L) In situ localization of *KN1* mRNA in *KN1*-N2 mutant leaves shows expression over developing vascular cells (arrowed); this expression is not seen in leaves of wild-type sibling plants (not shown). (M-O) Serial sections adjacent to the one shown in L probed with KNOX8, *RS1* or KNOX3 respectively; no expression of these genes is detected in knotted leaves. Bar, 50 μm.

veins of the leaf blade; KN1 is immunohistochemically undetectable in wild-type leaves (Smith et al., 1992). We reasoned that if *RS1* or KNOX3, which show expression in a subset of *KN1*-expressing cells, or KNOX8, which shows a similar pattern of expression to *KN1*, are directly regulated by *KN1*, then they might also be ectopically expressed in knotted leaves. However, in plants carrying two different dominant *KN1* alleles, *Kn1*-N2 (Fig 5K-O) and *Kn1*-Z3 (not shown) (Freeling and Hake, 1985), *KN1* mRNA is ectopically expressed in leaf vascular strands, as predicted, but no expression of the other 3 homeobox genes was detected in adjacent sections.

In summary, the expression of the four homeobox genes differs in the vegetative apex and meristem, whereas in inflorescence and floral meristems their expression is more similar. Although KNOX8, *RS1* and KNOX3 are expressed in some of the same cells which express *KN1*, we have found no evidence for direct interactions controlling their expression.

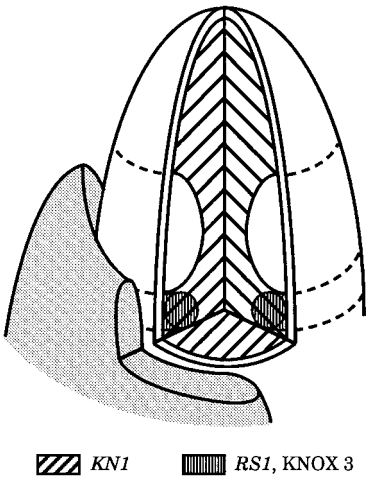

Fig. 6. Diagrammatic representation of the expression of *KN1*, *RS1* and KNOX3 in the vegetative meristem. Part of the meristem and P_1 leaf (stippled) has been cut away to show the presumed disc of leaf insertion of the P_0 leaf, which does not express *KN1*, and the ring of cells below, which also expresses *RS1* and KNOX3, and is the proposed incipient internode and axillary bud.

DISCUSSION

Plant development differs fundamentally from animal development, in that only a basic body plan is laid down during plant embryogenesis, and new organs are initiated throughout the plant life cycle. We show here, however, a common feature in plants and animals is that pre-patterns of homeobox gene expression predict morphogenetic events.

Morphological studies (Sharman, 1942) and clonal analysis (Johri and Coe, 1982; McDaniel and Poethig, 1988) have shown a lineage relationship in maize between the leaf and the internode and axillary bud below it, and together these make up a segmentation unit of the vegetative shoot or 'phytomer'. Using the different homeobox probes we can visualize domains within the organogenic zone of the meristem which will give rise to different parts of the segmentation unit. Absence of *KN1* expression marks the presumed position of the incipient (P_0) leaf, while *RS1* and KNOX3 are expressed in a ring of cells just below the P_0 leaf (summarized in Fig. 6). In the unexpanded shoot it is difficult to recognise the structures labeled by *RS1* and KNOX3. However the position of their expression in relation to the P_0 preprimordium and at the base of the disc of insertion of young leaves leads us to believe that this expression corresponds to the incipient internode and axillary bud (Sharman, 1942). This is supported by the visualization of *RS1* and KNOX3 expression in the developing internode and axillary bud near the base of older leaves; they may also be expressed in the leaf node which is morphologically indistinct in the unexpanded shoot. We conclude that *RS1* and KNOX3 expression in the meristem corresponds to incipient internode and axillary bud, which make up the basal limit of the segmentation unit of the shoot. Why only the periphery of the developing internode, and not the internal ground tissue, is labeled by *RS1* and KNOX3 is unclear, though this internal tissue is derived from a different part of the meristem, the rib zone.

It is interesting to speculate whether the earliest expression of *KN1, RS1* and KNOX3 precedes the determination of the position of a new primordium. This determination event has been estimated in *Lupinus albus* to occur about half a plastochron before the emergence of the primordium (Snow and Snow, 1933). In the examples shown here, one or two pericli-

nal divisions, which have been described as the first indication of the new primordium (Ledin, 1954; Esau, 1965), were visible in the tunica layer at the predicted position of the P_0. We have also occasionally observed two rings of cells that do not express *KN1* and two rings of *RS1* and KNOX3 expression above the P_1 leaf, suggesting that an even earlier differentiation event is marked by these homeobox genes. This suggests that these changes in gene expression patterns precede or at least are concomitant with the determination of the position of primordia in the meristem. Although a striped pattern of expression within the meristem is consistently observed for *RS1*, KNOX3 is often expressed diffusely throughout the meristem, though always in discrete stripes in the stem. It is possible that the stripe of KNOX3 expression in the meristem arises only at a specific stage during the plastochron.

How these patterns of homeobox gene expression arise in the meristem is unclear, though two major hypotheses have been forwarded to explain the positioning of primordia. The first suggests that inhibitory substances are responsible for positioning the new primordium at a given spacing relative to preexisting primordia (e.g. Wardlaw, 1949). The second hypothesis suggests that reorientation of cell wall cellulose microfibrils allows the change in growth polarity required for outgrowth of the primordium (e.g. Green, 1985). If the genes discussed here are indeed involved in determining the position of primordia in the meristem, then presumably their expression is in response to one or both of these factors.

In contrast to the differing expression patterns in the vegetative shoot meristem, these homeobox genes are all expressed uniformly throughout inflorescence and floral meristems. We view the uniform expression in the inflorescence meristem as consistent with our hypothesis about the expression patterns in the vegetative meristem. The products of the inflorescence meristem, branch primordia, are also indeterminate. Therefore all cells in the inflorescence meristem and branch primordia, being indeterminate, are labeled by *KN1*, and since cells of the

inflorescence meristem will form lateral indeterminate buds or inflorescence shoot internode, they are also all labeled by *RS1* and KNOX3. However, it is unclear why the patterns of *RS1* and KNOX3 expression do not revert to stripes in floral meristems. This could be due to the compressed nature of the floral meristem, or may reflect differences in the state of determination of floral and vegetative meristems. None of the homeobox genes is expressed in the root apical meristem, suggesting that they are involved in functions specific to the shoot. This may reflect basic differences in the properties of the root and shoot apices, one of which is that lateral organs are initiated from the shoot meristem, but not from the root meristem.

Since the expression domains for these genes are similar, we were interested in the possibility that their expression might be controlled by direct regulatory interactions, as has been documented for homeobox genes controlling *Drosophila* development (e.g. Ingham, 1988). We therefore looked to see if KNOX8, *RS1* or KNOX3 are expressed in knotted mutant leaves, where KN1 protein is ectopically expressed (Smith et al., 1992). Whereas ectopic expression of *KN1* mRNA was seen in knotted leaves, no ectopic expression of the other genes is observed. A negative result does not disprove an interaction, however, since other factors that may be absent from leaves are probably required for the expression of these genes in the meristem.

KN1 mRNA is not detected in the tunica layer of meristems, although KN1 protein is present. We do not believe that these observations are artefactual, since ubiquitin mRNA is detected in the epidermis, and we have detected *KN1* mRNA in the epidermis of tobacco plants overexpressing the *KN1* cDNA under the control of the cauliflower mosaic virus 35S promoter (D. J., R. Williams and N. Sinha, unpublished data). Additionally we do not believe that this observation could be explained by cross reaction of the polyclonal antibody with another homeodomain protein, since two different anti-KN1 monoclonal antibodies, each of which recognize a part of KN1 outside of the conserved ELK-homeodomain part of the protein (Vollbrecht et al., 1993; D. J. and L. Smith, unpublished data), also label the nuclei of cells in the tunica. Whilst one explanation for the discrepancy could be cell-specific differences in stability of *KN1* mRNA, another hypothesis is relevant, since a signal has been proposed to emanate from *KN1*-expressing cells. Clonal analysis showed that for knot formation to occur in leaves, the *KN1* product is required only in the vascular/inner mesophyll layer, although changes in cell division and differentiation occur in all cell layers (Gelinas et al., 1969; Hake and Freeling, 1986; Sinha and Hake, 1990). It was proposed that a signal moves from the vascular/inner mesophyll layer to instruct the epidermis and other layers to divide. Assuming the observations described here are not artefactual, we hypothesize that KN1 protein itself could be the signal that moves between cells, though this movement would have to be short range or selective, since the protein does not move into the P_0 or young leaf primordia, and in knotted leaves does not move to adjacent veins. Whilst movement of transcription factors between cells has not been previously described, such a mechanism was also hypothesized for the establishment of stripes of *hairy* gene expression in the cellularized growth zone of the short germ band *Tribolium* embryo (Sommer and Tautz, 1993).

The expression patterns presented here suggest a common function of homeobox genes in the animal and plant kingdoms. In animals, homeobox genes are required for setting anterior-posterior coordinates in both segmented and non-segmented systems (e.g. Kenyon and Wang, 1991; Dolle et al., 1989). Even though plants and animals diverged at the unicellular stage, there may be some property of homeobox genes which made them well suited for the role of defining cell fate and for defining boundaries in morphogenetic fields during the evolution of multicellular organisms. Three dominant leaf mutations in maize, *Kn1, Rs1* and *Lg3* have now been found to be caused by lesions in homeobox genes (Freeling, 1992). The results presented here and elsewhere (Smith et al., 1992, Becraft, Schneeberger, Hake and Freeling, unpublished data) indicate that several members of this class of homeobox genes are not normally expressed in leaves, though the dominant leaf phenotypes of mutations in these genes and their restricted expression patterns in the meristem suggest roles in the control of cell fate.

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